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(54) Mutant human growth hormones and their uses

(57) In accordance with the present invention, there are provided mutant human growth hormone proteins which exhibit enhanced affinity for growth hormone but lowered hormone activity, base sequences encoding the same and their production processes as well as uses of said proteins. The proteins according to the present invention, with their enhanced affinities for the growth hormone receptor, can inhibit the binding of growth hormone to its receptor, while they retain lowered growth hormone activities, thus finding application as a medicament for the treatment of acromegaly and gigantism.

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Description

The present invention relates to a mutant human growth hormone protein showing an amino acid sequence given in Fig. 1, to a deoxyribonucleotides showing a base sequence which encodes said amino acid sequence, to mutant human growth hormone proteins each showing an amino acid sequence which has its amino acid residue moiety subjected to partial replacement, insertion or depletion to such an extent as may not cause loss of its characteristic features that enhanced affinity for the growth hormone receptor is exhibited and that decreased growth hormone activity is retained, and to their uses in the manufacture of medicaments for the treatment of gigantism and acromegaly.

The biologically inactive human growth hormones according to the present invention act as an antagonist of normal growth hormone for its receptor to thereby inhibit the disturbances and excessive growth caused by oversecretion, and can be utilized as a medicament with improved safety for the treatment of gigantism and acromegaly.

As the genetic disorders brought about by growth hormone, there are known growth retardation due to a deficiency of growth hormone as well as gigantism and acromegaly owing to excessive expression. For the growth hormone deficiency, supplementation therapy with growth hormone has been in wide use, but no effective drug has been developed so far for the treatment of gigantism and acromegaly.

In 1978, Kowarski et al. reported for the first time the discovery of a biologically inactive growth hormone (a mutant growth hormone) (Kowarski, A. A. et al., J. Clin. Endocrinol. Metab., 47: 461, 1978). However, understanding of the mutant growth hormone at the molecular level has not yet been elucidated up to now, although there was published a report that an abnormal polymer of growth hormone was identified in the blood from a child with dwarfism (Valena, L. J. et al., N. Engl. J. Med., 312:214, 1985). A child, who was found to contain a biologically inactive growth hormone in the circulatory blood, showed a high blood level of a mutant growth hormone but a low blood concentration of insulin-like growth factor (IGF-1). thereby causing retarded growth and development. However, such growth retardation is characterized by good response to normal growth hormone administered (Hayek A. et al., Pediatr. Res., 12: 413, 1973; Rudman, D. et al., N. Engl. J. Med., 305: 123, 1981; Plotnick, L. P. et al., Pediatrics 71: 324, 1983; Bright, G.M. et al., 71:576, 1983).

In recent years, progresses in protein engineering and genetic engineering have enabled structural research to be conducted on the binding of hormones to their receptors as well as the elicitation of their activities, and as a result, the causes for various genetic diseases have been clarified.

Cunningham et al. prepared a number of human growth hormone variants by using protein engineering procedures to conduct investigation on their binding sites for the growth hormone receptor, and as a result, identified the region being involved in the binding of growth hormone to the receptor, which constitutes a region consisting of the amino-terminal (2-19) amino acid residue, the carboxy-terminal (54-74) amino acid terminal and the carboxy-terminal (167-191) amino acid residue (Cunningham, B.C. et al., Science 243: 1330, 1989).

Furthermore, Uchida et al. prepared growth hormone variants having amino acid residues subjected to different replacements to thereby measure their differentiating activities for 3T3-F 442A cells, leading to the suggestion that the amino acid sequence 62 to 67 region is of critical importance to the development of biological activity (Uchida et al., Biochem. Biophys. Res. Commun., 172: 352, 1990).

Recently, a crystallographic study yielded a remarkable finding on the mode of binding of human growth hormone to its binding protein (a portion of the receptor protein) (De Vos A.M. et al., Science 255: 306, 1992); it is assumed that growth hormone binds consecutively to the growth hormone receptor in a manner where the domain 1 of growth hormone in the first place binds to the first growth hormone receptor and then the second domain 2 of growth hormone binds to the second growth hormone receptor, resulting in the formation of a dimer of the growth hormone receptor, whereupon signals of growth hormone are transmitted into cells.

Interesting among others is the fact that although the domain 1 of human growth hormone differs in amino acid residue from the domain 2, the binding sites of the receptor protein show the common amino acid residue. It was also recognized that growth hormone variants produced by protein engineering techniques binds competitively to the receptor (Fuh G. et al, Science, 256: 1677, 1992).

Recent progresses in gene analysis have made it feasible to identify the abnormal genes being contributed to a large number of genetic diseases. This is the case with the gene for growth hormone which brings about dwarfism, as well. Since growth hormone develops its physiological activity as mediated by the receptor on the cellular membrane, genetic abnormalities associated with growth hormone can roughly be divided into two groups, abnormality in receptor gene and the one in growth hormone itself.

Because growth hormone gene exists on the autosome, furthermore, its abnormalities are known to assume the form of recessive inheritance. In order to allow phenotypic expression of such abnormalities, consequently, it is required that abnormalities are brought about simultaneously in the alleles of the parent.

In the past, there have been reported many cases of growth retardation resulting from the complicated combination of mutations in the parent's growth hormone genes, such as whole depletion, partial depletion and base replacement. When either of the parent is normal, the mutant growth hormone is known to stay inside the intracellular secretory

granules.

However, detailed investigation has not yet been conducted on the analysis at the molecular level of mutant growth hormones generated by missense mutation in the living body, as well as its role to be played in the living body. Neither known has been any effective method to suppress the overaction of growth hormone.

The present inventor found that a 5-years old boy with dwarfism having a delayed bone age showed a high serum concentration of growth hormone and, in the induction test, retained a lowered level of IGF-1, though he exhibited an increased serum concentration of growth hormone, and this finding, followed by further subsequent research, culminated into the present invention.

It seemed likely that this endocrinological finding is consistent with the phenomena noted in the growth hormone insensitivity syndrome (Rosenbloom, A. L., Acta Pediatr. Scand. (Suppl), 383: 117, 1992).

However, consecutive administration of growth hormone brought about a significant improvement in growth of the patient, which excluded the possibility of diagnosing it as the Laron type syndrome, because Laron-type dwarfism is caused by the disorders of growth hormone receptors.

The present inventor, using the Nb2 bioassay method, discovered that the serum growth hormone found in the children suffering from this sort of disorders is an inactive type growth hormone, unlike the one secreted by normal children, and also identified the hormone as a mutant growth hormone by use of isoelectric focusing.

The mutant growth hormone was found to undergo replacement of the arginine residue with the cysteine residue (R→C) at codon 77 of growth hormone (Fig. 1). The site of replacement is located in the second α-helix of growth hormone, behind a site 1 of binding to the receptor (Cunningham, B.C. et al., Science, 254:821, 1991). The substituted cysteine is assumed to form a new disulfide bond and cause the resultant molecule to change the charge, and this brings about conformational alterations, resulting in generation of a mutant growth hormone with reduced growth-hormone activity.

In the intracellular signal transduction of growth hormone, dimerization of the growth hormone receptors through ligand bonding and phosphorylation of the tyrosine residue in their proteins are considered crucially important (Argetsinger, L.S. et al., Cell, 74: 237, 1993: Silva, C. M. et al.: J. Biol. Chem., 269: 27532, 1994).

The growth-hormone binding protein is located in the extracellular domain and functions as a growth hormone reservoir in serum in vivo (Herington, A. C. et al., Acta Endocrinol. (Copenh), 124: 14, 1991).

The affinity of the mutant growth hormone for the growth-hormone binding receptor was found to be about 6 times greater than that of the wild-type one (Figs. 7 and 8), suggesting that the domains 1 and 2 in the mutant growth hormone show different affinities for the receptor from those in the wild-type one. The biological characteristic of the mutant growth hormone lies in markedly lowered activity of cellular signal transduction developed through phosphorylation of the receptor, despite its greater affinity for the receptor protein.

Wild-type growth hormone, after administered to the patient consecutively for 3 days, did not give rise to conspicuous response to IGF-1, whereas it, when given to the patient over a prolonged period of time, acting as an antagonist to suppress the secretion of endogenous mutant growth hormone as well as its binding to the receptor, was found to be effective in increasing the plasma concentration of IGF-1 and in improving the growth and development.

Consequently, these findings led the present inventor to the conclusion that the mutant growth hormone, when administered to patients with gigantism or acromegaly caused by oversecreted growth hormone, may act as an antagonist to suppress their excessive growth.

The present invention has been completed on the basis of the above novel findings and relates to (1) a mutant human growth hormone protein showing an amino acid sequence given in Fig. 1, (2) a deoxyribonucleotides showing a base sequence which encodes said amino acid sequence, (3) mutant human growth hormone proteins each showing an amino acid sequence which has its amino acid residue moiety subjected to partial replacement, insertion or depletion to such an extent as may not cause loss of its characteristic features that enhanced affinity for the growth hormone receptor is exhibited and that decreased growth hormone activity is retained, and (4) uses thereof.

The mutant human growth hormones of the present invention, because of their endogenous identity, do not exert any adverse effects to the living body, while they only induce growth retardation, and can therefore find application as an effective medicament for the treatment of gigantism and acromegaly, against which diseases no therapeutic agent has been developed in the past. The mutant growth hormones exhibit about 6 times greater receptor affinity and are useful as a medicament for the treatment of gigantism at doses equal to or smaller than the dose employed in the treatment of dwarfism.

In accordance with the known art, it is easily and practically feasible to subject the DNA of the novel mutant growth hormone of the present invention to partial depletion, insertion or substitution of nucleotides to thereby produce growth hormone variants showing enhanced receptor affinity but substantially being free from hormone activity, as being exemplified by the sequences illustrated in Figs. 2 and 3. By using the protein engineering techniques, furthermore, it is possible not only to identify the site of binding of the mutant growth hormone to the receptor but also to produce the peptide of such binding site to thereby utilize the same as a medicament for the treatment of gigantism and acromegaly.

The novel mutant growth hormone of the present invention can be produced by linking the hormone encoding DNA

to a reproducible plasmid, then transforming cells with the plasmid and cultivating these host cells. Such host cells include bacteria, yeasts and animal cells.

Prokaryotic cells, such as bacteria, are suited for cloning of desoxyribonucleotides. For example, plasmid pBR 322 derived from *E. coli* contains a gene being resistant to ampicillin or tetracycline, and provides a practical means for identifying the resultant transformed cells. Furthermore, bacterial plasmids contain promoters which can function and operate in the expression of their own proteins.

In addition to prokaryotic cells, eukaryotic cells inclusive of yeasts are of use, as well, and plasmid YRp7 is employable commonly in the expression in *Saccharomyces,* a strain of yeasts (Stinchcomb et al., Nature, 282: 39, 1979).

Animal cells are also utilized as a host cell, and their cell lines include, for example, Hela cells, CHO (Chinese hamster ovary) cells, COSM6 and COS-7, whereby the promoters of polyoma viruses, adenovirus 2, cytomegaloviruses and simian viruses serve a useful purpose to act to control the expression plasmids of such cell lines (Thomsen et al., PNAS, 81: 659, 1984).

Animals can be immunized with the mutant growth hormone or its variants to thereby produce their antibodies. Additionally, animals can be immunized by the known techniques to prepare monoclonal antibodies from cells capable of secreting specific antibodies.

In accordance with the present invention, it is facilitated to prepare the mutant growth hormone and its variants in large quantities, and there can be provided their better understanding at the molecular level, which renders it feasible to develop therapeutic and diagnostic agents for the diseases associated with growth hormone. This includes the preparation of drugs for gene therapy, which offer the essential treatment method for such diseases.

The nucleotides for the mutant growth hormones or the nucleotide for the binding-site protein can be incorporated into suitable vectors inclusive of virus vectors from retroviruses, adenoviruses, etc. and this affords a possibility of using them as a drug for gene therapy for gigantism and acromegaly.

Below described is an example to illustrate the present invention in more detail, with reference to the attached drawings, in which:

Fig. 1 is an amino acid sequence of the mutant growth hormone (the 77-position, R→C) obtained in Example 1 and a base sequence encoding its protein.

Fig. 2 is an amino acid sequence of the mutant growth hormone having undergone mutation through substitution (the 53 position, C→A) obtained in Example 1 and a base sequence encoding its protein.

Fig 3 is an amino acid sequence of the mutant growth hormone (the 165 position, C→A) having undergone mutation through substitution obtained din Example 1 and a base sequence encoding its protein.

Fig. 4a is a genetic structure of the mutant growth hormone and a design of a primer for PCR amplification; Five exxon sites are indicated by the box, while the PCR primer by the arrow;

Fig. 4b is a photograph showing a DNA sequence of the mutant growth hormone, where alteration of arginine to cysteine at codon 77 is indicated, as was determined by direct sequence analyses of genome DNA and RNA by use of PCR.

Fig. 5 is a flow sheet for the construction of cDNA for alteration of cysteine at 53 or 165 position to alanine, with the oligonucleotides showing the following sequences being used as a primer:

f1: 5'ACAGAAACAGGTGGGGCAA3'
R2: 5'AATAGACTCTGAGAAAGCGGG3'
R3:5'GTCCATGTCCTTCCTGAAGGCGTAGAG3'

PCR amplification was performed using the primers, f1 and R2, for the mutation at the position 53 (C→A), while using the primers, f1 and R3, for the mutation at the position 165 (C→A). Separately, there were prepared the portions downstream of cDNA of normal growth hormone after having been digested with the restriction enzymes Hinfl and NlaIII, respectively, followed by binding to the PCR products with a ligase.

Fig. 6 is a graph showing the results of isoelectric focusing (IFE) of the mutant and wild growth hormones in serum. Serum samples (150 to 300 ul) was subjected to isoelectric focusing in 1 % HPMC-4 % Ampholine (pH 3.5 - 8.0), and the sample fractions were separated, pooled and assayed for immuoactivity for growth hormone (■), with the pH gradient formed during IEF being designated by (♦). The mutation through substitution of cysteine for arginine is assumed to bring about an isoelectrical decrease by pH 0.16. The peaks for wild type and mutant growth hormones are designated by the white and black arrows, respectively. a; Proband (patient: a boy) b: father

Fig. 7a is a graph showing how wild type and mutant growth hormones inhibited binding of [^{125}I]-labeled human growth hormone to IM-9 cells.

Cells (IM-9) at the final concentration of 1-3 x 10^7 /ml were incubated, while adding wild-type and mutant growth hormones at increased concentrations in accordance with their addition-concentration dependencies: 0.8 ng/ml of [^{125}I]-labeled human growth hormone (Du'Pont, USA) (0.33 uCi/ml), 250 ul of the total solution, 30°C. After cultivation

for 4 hours, the cells were collected, washed and assayed for radioactivity bound to the cells.

Fig. 7b is a graph showing inhibition of binding of [^{125}I]-labeled human growth hormone to the growth-hormone bidning protein.

[^{125}I]-Labeled human growth hormone (0.6 uCi/ml), recombinant human growth hormone binding protein (0.6 nM) and anti-growth hormone receptor mouse clonal antibody (Mab 263; AGEN, Australia) (1 : 100,000) were cultivated at 4°C for 16 hours, while increasing the respective concentrations of wild-type and mutant growth hormones, followed by addition of 10 % anti-mouse IgG (goat) antibody (50 ul), 1 % normal mouse serum (50 ul) and 5 % PEG (300 ul). The reaction solution was cultivated at 4°C for further 4 hours and centrifuged, and the precipitate (pellets) was assayed for radioactivity by a gamma-counter, with a mean for three measurements being indicated.

Fig. 8a is a photograph of an electrophoretic pattern showing a tyrosine phosphorylation in IM-9 cells being dependent on wild-type and mutant growth hormones.

IM-9 Cells were treated at 37°C for 15 min in the presence and absence of 100 ng/ml of human growth hormone (Lanes 1 and 2); in the presence of wild-type growth hormone (Lane 3: 10 ng/ml, Lane; 100 ng/ml), mutant growth hormone (Lane 5; 10 ng/ml, Lane 6; 100 ng/ml) and mutant growth hormone of a constant concentration of 10 ng/ml, with increasing concentrations of wild-type growth hormone (Lane 7; 10 ng/ml, Lane 8; 25 ng/ml, Lane 9; 50 ng/ml, Lane 10; 100 ng/ml), respectively. Detergent lysates of these cells were immunoprecipitate with a phosphorylation-tyrosine specific antibody and analyzed by Western blotting with the same antibody conjugated to horseradish peroxidase. The molecular weights in unit of kilo-daltons were indicated on the left margin.

The symbols "arrow" designate the tyrosine-phosphorylated protein bands produced through action of growth hormone.

Fig. 8b is bar graphs showing the results of densitometry analysis for anti-phopshorylated tyrosine immunoblotting of p 120.

The amount of tyrosine-phosphorylated p-120 (IM-9 cells reported as JAK2) was determined by densitometry. Intensity of densitometry is expressed in relation to the one obtained as a control treated without growth hormone. Indicated is a mean (± SEM) for found values from three independent experiments, with statistical analysis being conducted by Student's t-test.

Example 1:

The following investigation was carried out on the blood samples drawn from the above-mentioned 5-years old boy with dwarfism showing a delayed bone age:

Hormone-assaying method

A serum concentration of growth hormone was analyzed with use of an immunoradiometric assay kit manufactured by Pharmacia of Sweden, and biological activity of growth hormone was measured by the slightly modified Tanaka et al. (Tanaka T. et al., J. Cli. Endocrinol. Metab., 51: 1058, 1980) method, whereby in the Nb2 bioassay method, rabbit antiserum (NIDDK-anti-hORK-IC5; NIH) to human prolactin (hPRL) was added in a 100,000-fold dilution to inhibit through neutralization the growth-stimulating activity of human prolactin. By these procedures, the serum growth hormone was measured and analyzed with the patient with dwarfism and normal subjects as a control.

Isoelectric focusing

Isoelectric focusing was performed by using the Tsventnitsky et al. (Tsventnitsky V. et al., Biochem. J., 307: 239, 1995) method; serum samples were electrofocused for separation with 1 % HMPC (hydroxypropyl methylcellulose) - 4 % ampholine buffer at a pH gradient of pH 3.0 to 8.0 to thereby collect different fractions for the analysis of immunoactive growth hormone. Pooled serum samples from 10 normal subjects were used as a control.

Isolation and genetic analysis of the gene for the mutant growth hormone

Genomic DNA was isolated from peripheral-blood leukocytes (Gross-Bellard M. et al., Eur. J. Biochem., 36: 32, 1973), and amplified by the PCR method (Fig. 4). The oliognucleotides, namely F3; 5'TATGAATTCCTCTGCCTGCCCT-GCC TCAAGAG3', GAD:5'CTAACACAGTCTCTCAAAGT3', GSD:5'ACTTTGAGA GACTGTGTTAG3', GAE : 5'TGGAGTGGCAACTTCCAGGG3' and GHS1: 5'CTCAGGGTCCTGTGGACAGCTCACCTAGCTGCA3', were used as a promoter for the amplification of the genomic DNA.

The PCR amplification was performed by the following procedure: with F3-GAD and GHS1-GAD, the first denaturation was effected at 92°C for 3 min, followed by 35 cycles consisting of one minute of denaturation at 92°C, 2 minutes of annealing at 60°C and 2 minutes of extension at 72°C, with the final cycle extension at 72°C being performed

for 7 min, and with GSD-GAE, the cycle consisting of one minute of denaturation at 92°C, 2 minutes of annealing at 60°C and 2 minutes of extension at 72°C was repeated 35 times, with only the final cycle extension being performed for 7 min.

The amplification products were extracted, then subcloned into pBS SK(+) (Stratagene, USA) or pT7blue (Novagem, USA) and sequenced with use of 373A DNA Sequencer (Perkin Elmer, USA). Furthermore, the site (Arg→Cys) of mutation in the DNA of the patient was identified, and the resultant PCR product was subjected to direct DNA sequencing with use of a double-strand DNA cycle sequencing kit (Gibco BRI, USA) in order to exclude a possibility of undergoing any misreactions in the PCR reaction. As a result, it was found that the patient's DNA had undergone substitution the arginine residue at the 77 position with a cysteine residue (Fig. 1).

RNA Analysis

Lymphocytes were separated by use of MPRM Ficoll-Hypaque (Flow Lab., USA), and total RNA was isolated by the conventional means (Maniates T. et al., cold Spring Harbor Laboratory Press, 1982). cDNA was synthesized with 1 μg of RNA (Martynoff G. et al., Biochem. Biophys, Res. Commn., 93: 645, 1980), an the synthesized cDNA was used in the PCR reaction to amplify cDNA for the growth hormone gene. GHS2; 5'TGGACAGCTCACCTAGCTGCA3', GHAS1; 5'GGATTTCTGTTGTGTTTCCT3', GHS3; 5'TTGACACCTACCAGGAGTTT3' and GHAS3; 5'CTAGAAGCCACAGC TGCCCT3' were used as a oligonucleotide primer to perform the PCR amplification under the following conditions:

With GHS2-GHAS1, denaturation was effected at 92°C for 3 min, and the cycle consisting of one minute of denaturation at 92°C , 1.5 minutes of annealing at 68°C and 1.5 minutes of extension at 72°C was repeated 40 times, with the final cycle extension being performed for 7 min.

With GHS3-GHAS3, the first denaturation was effected, followed by 40 cycles consisting of one minute of denaturation at 92°C , 1.5 minutes of annealing at 68°C and 1.5 minutes of extension at 72°C was repeated 40 times, with the final cycle extension being performed for 7 min., and the amplified products were subjected to base sequencing.

Construction of cDNAs for wild-type and mutant growth hormones

cDNAs of two types of human growth hormone, wild-type and mutant-type, were amplified by PCR, while using a cDNA library prepared from human growth hormone producing pituitary adenoma cells (Clontech, USA). and the accuracy each of the identified structures for growth hormone cDNAs was confirmed by base sequencing for DNA.

Referring to the oligonucleotide primers used in the PCR procedures, GHS1 was utilized as a sense primer, while 5'TAAGAATTCGAGGGGTCACAGGGATGCCACCCC3' employed as an antisense primer.

PCR was performed under the reaction conditions: the first denaturation was effected at 92°C for 3 min, and the cycle consisting of one minute of denaturation at 92°C , 1.5 minutes of annealing at 48°C and 1.5 minutes of extension at 72°C was repeated 40 times, with the final cycle extension being effected for 7 min.

cDNA of the mutant growth hormone was constructed with use of Transformer MT (Clontech, USA). To remove the signal sequence of cDNA of growth hormone, PCR amplification was conducted with a sense primer (5'GCGGATCCTTCC CAACCATTCCCTTATC3') containing a BamH1 site incorporated artificially and GHAS1 as an antisense primer. The resultant cDNA was determined for base sequence by the direct base sequencing method to confirm the mutation (Fig. 1).

Expression and functional analysis of wild-type (normal) and mutant growth hormones

Each of the expression vectors for the production of wild-type and mutant growth hormones comprised a DNA sequence containing promoter operator PLOL derived from λ-bateriophage, a DNA sequence containing a N-utilization site capable of binding the anti-transcription terminating factor N-protein produced by host cells and a ribosome binding site capable of binding mRNAs of wild-type and mutant growth hormones to the ribosome inside host cells, ATG initiation codon and a restriction enzyme site for inserting the desired gene into the vector in phase with the ATG initiation codon ATG (Japanese Patent Publication No. 87780/ 1994).

The expression vectors were introduced into suitable host cells containing non heat-resistant repressor C1, for example, *E. coli.,* and allowed to express wild-type and mutant growth hormones, respectively, when the host cells were heated at the repressor demolition temperature. Such expression products held at the amino terminal the methionine residue derived from the initiation codon, but elimination of such methionine residue with a specific aminopeptidase can yield the matured wild-type and mutant human growth hormones (Japanese Unexamined Patent Publication No. 500003/1982).

The transformed cells were cultivated, and the cell suspension was subjected to centrifugation or filtration to collect the cells, followed by lysis by means of physical and chemical techniques to isolate the mutant growth hormone.

The purification procedure was carried out by combinations of the known procedures, fractionation with ammonium sulfate, etc., gel filtration chromatography, ion exchange chromatography, affinity chromatography with use of antibody and normal-phase or reverse-phase high performance chromatography.

In order to prepare small-amount samples for experimental uses, cDNAs of wild-type and mutant growth hormones were cloned individually into a BamHI-EcoRI site of a plasmid (pGEXKG) and then incorporated into DH5α cells. The expression products were also prepared in the cell line fused with the glutathione-S-transferase gene supplied by Pharmacia of Sweden.

It was suggested that the intramolecular crosslinking between two cysteines within the recombinant mutant growth hormone obtained by the above procedures occurs in three different types, i.e. normal type (53-165) as well as two mutant types (53-77) and (77-165). Accordingly, cDNA prepared from lymphocytes of the patient was subjected to replacement for mutation by the procedure as shown in Fig. 5 to thereby produce cDNA in which cysteine at the 53 or 165 position was substituted with an alanine residue. In this case, there can be produced cDNA in which the cysteine at the 53 or 165 position is substituted with a serine residue.

These genes were expressed in *E. coli* to produce two kinds of mutant growth hormones in which a pair of cysteines formed crosslinking at the 77 and 165 positions (Fig. 2) and at the 53 and 77 positions (Fig. 3), respectively.

The bioactivity each of wild type and mutant growth hormones was determined by the IRMA and Nb2 bioassay system. The Nb2 bioassay was performed in the presence or absence of serum from the patient who showed neither growth hormone nor prolactin detected. Recombinant human growth hormone binding protein (rhGHBP) was added individually to the samples to the final concentrations of 0.1, 0.5 or 1 nM.

Competitive binding was studied in the human lymphoblastoma cell line IM-9 capable of expressing growth hormone receptor by the one-step receptor analysis method (Lesniak, M. A. et al., J. Biol. Chem., 249: 1661, 1974).

Direct binding of wild-type and mutant growth hormones to rhGHBP was investigated by use of immunoprecipitation.

Growth-hormone dependent tyrosine phosphorylation in IM-9 cells was detected by the Silva et al. method (Silva, M.D. et al., Endocrinology, 132: 101, 1993). Antiphosphorylation tyrosine monoclonal antibody (RC20: Transcution Laboratories, USA) was used in the immunoprecipitation and western blotting procedures, and antibody binding was visualized with an ECL kit manufactured by Amersham Co. of USA.

Isoelectric focusing demonstrated that in addition to the known wild type (normal) growth hormone, the mutant growth hormone was present in serum of the proband (patient) (Fig. 6).

In order to estimate whether or not the mutant growth hormone gene is bioreactive, the genes of the mutant and wild-type growth hormones were expressed in cells transformed with the expression vector possessing a promoter operator derived from λ-phages to thereby give the products, while the genes were also expressed in the GST fused protein system to obtain the products.

Both of the mutant and wild-type growth hormones were found to be immunoreactive by assay in IRMA cells. Their bioactivities were also measured by the Nb2 bioassay.

Despite the fact that both substances were found to exhibit a similar degree of bioactivity in the NB2 bioassay in a serum-free medium, the bioactivity of the mutant growth hormone decreased to less than half that of wild-type growth hormone in the patient's serum medium. In anticipation of the possibility of interference being caused by the growth hormone binding protein in the Nb2 bioassay system, the recombinant growth-hormone binding protein was added to the assay medium.

A ratio of bioactivity to immunoreactivity of the mutant growth hormone was found to decrease markedly to 0.45 ± 0.05 ($p<0.05$) and 0.22 ± 0.08 ($p<0.05$) in the presence of 0.5 nM and 1 nM of the recombinant growth-hormone binding protein, respectively, Such concentrations of the protein correspond to those of the actual physiologic binding protein in the peripheral blood.

Binding of [^{125}I]-labeled human growth hormone to human growth-hormone receptor in IM-9 cells was found to change in a concentration-dependent manner through replacement switching from wild-type to mutant growth hormones. The replacement with the mutant growth hormone exhibited a shoulder at the protein concentration in the range of 10^{-11} to 10^{-9} M.

Their individually found IC_{50} values were almost equal, being at 0.84 ± 0.30nm and 0.86 ± 0.41nm, respectively.

However, the replacement with the mutant growth hormone did not proceed smoothly at the protein concentration in the range of 10^{-11} to 10^{-9} M (Fig. 7).

In addition, binding of [^{125}I]-labeled human growth hormone to the recombinant human growth-hormone binding protein in IM-9 cells was found to change in a concentration-dependent manner through replacement switching from wild-type to mutant growth hormones, as well.

The mutant growth hormone showed IC_{50} of 0.12 ± 0.02 nM (mean ± SE, for 3 measurements) being remarkably lower than the counterpart of 0.68 ± 0.08 nM for wild type growth hormone, and demonstrated about 6 times greater affinity for the binding protein than wild-type one.

Growth-hormone dependent tyrosine phosphorylation in the growth hormone receptor with use of IM-9 cells was

compared between wild-type and mutant growth hormones by means of Western blotting.

In contrast with the fact that both recombinant growth hormone and wild-type growth hormone, namely normal growth hormone, promoted tyrosine phosphorylation, the mutant growth hormone not only failed to exert any action on the tyrosine phosphorylation by itself but also inhibited markedly the phosphorylation induced by wild-type growth hormone. Inhibition of tyrosine phosphorylation was observed even when the mutant growth hormone was added simultaneously with wild-type growth hormone at a concentration of 1 : 10 (Fig.8).

The antibodies according to the invention preferably immunoreact with the mutant growth hormones of the invention preferentially or exclusively as compared to the wild-type growth hormone.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

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(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 576 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

```
TTCCCAACCA TTCCCTTATC CAGGCCTTTT GACAACGCTA TGCTCCGCGC CCATCGTCTG      60
CACCAGCTGG CCTTTGACAC CTACCAGGAG TTTGAAGAAG CCTATATCCC AAAGGAACAG     120
AAGTATTCAT TCCTGCAGAA CCCCCAGACC TCCCTCTGTT TCTCAGAGTC TATTCCGACA     180
CCCTCCAACA GGGAGGAAAC ACAACAGAAA TCCAACCTAG AGCTGCTCTG CATCTCCCTG     240
CTGCTCATCC AGTCGTGGCT GGAGCCCGTG CAGTTCCTCA GGAGTGTCTT CGCCAACAGC     300
CTGGTGTACG GCGCCTCTGA CAGCAACGTC TATGACCTCC TAAAGGACCT AGAGGAAGGC     360
ATCCAAACGC TGATGGGGAG GCTGGAAGAT GGCAGCCCCC GGACTGGGCA GATCTTCAAG     420
```

CAGACCTACA GCAAGTTCGA CACAAACTCA CACAACGATG ACGCACTACT CAAGAACTAC 480

GGGCTGCTCT ACTGCTTCAG GAAGGACATG GACAAGGTCG AGACATTCCT GCGCATCGTG 540

CAGTGCCGCT CTGTGGAGGG CAGCTGTGGC TTCTAG 576

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 576 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

TTCCCAACCA TTCCCTTATC CAGGCCTTTT GACAACGCTA TGCTCCGCGC CCATCGTCTG 60

CACCAGCTGG CCTTTGACAC CTACCAGGAG TTTGAAGAAG CCTATATCCC AAAGGAACAG 120

AAGTATTCAT TCCTGCAGAA CCCCCAGACC TCCCTCGCTT TCTCAGAGTC TATTCCGACA 180

CCCTCCAACA GGGAGGAAAC ACAACAGAAA TCCAACCTAG AGCTGCTCTG CATCTCCCTG 240

CTGCTCATCC AGTCGTGGCT GGAGCCCGTG CAGTTCCTCA GGAGTGTCTT CGCCAACAGC 300

CTGGTGTACG GCGCCTCTGA CAGCAACGTC TATGACCTCC TAAAGGACCT AGAGGAAGGC 360

ATCCAAACGC TGATGGGGAG GCTGGAAGAT GGCAGCCCCC GGACTGGGCA GATCTTCAAG 420

CAGACCTACA GCAAGTTCGA CACAAACTCA CACAACGATG ACGCACTACT CAAGAACTAC 480

GGGCTGCTCT ACTGCTTCAG GAAGGACATG GACAAGGTCG AGACATTCCT GCGCATCGTG 540

CAGTGCCGCT CTGTGGAGGG CAGCTGTGGC TTCTAG 576

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 576 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

```
TTCCCAACCA TTCCCTTATC CAGGCCTTTT GACAACGCTA TGCTCCGCGC CCATCGTCTG    60
CACCAGCTGG CCTTTGACAC CTACCAGGAG TTTGAAGAAG CCTATATCCC AAAGGAACAG   120
AAGTATTCAT TCCTGCAGAA CCCCCAGACC TCCCTCTGTT TCTCAGAGTC TATTCCGACA   180
CCCTCCAACA GGGAGGAAAC ACAACAGAAA TCCAACCTAG AGCTGCTCTG CATCTCCCTG   240
CTGCTCATCC AGTCGTGGCT GGAGCCCGTG CAGTTCCTCA GGAGTGTCTT CGCCAACAGC   300
CTGGTGTACG GCGCCTCTGA CAGCAACGTC TATGACCTCC TAAAGGACCT AGAGGAAGGC   360
ATCCAAACGC TGATGGGGAG GCTGGAAGAT GGCAGCCCCC GGACTGGGCA GATCTTCAAG   420
CAGACCTACA GCAAGTTCGA CACAAACTCA CACAACGATG ACGCACTACT CAAGAACTAC   480
GGGCTGCTCT ACGCCTTCAG GAAGGACATG GACAAGGTCG AGACATTCCT GCGCATCGTG   540
CAGTGCCGCT CTGTGGAGGG CAGCTGTGGC TTCTAG                              576
```

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

```
ACAGAAACAG GTGGGGGCAA    20
```

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: HOMO SAPIENS

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

```
AATAGACTCT GAGAAAGCGG G    21
```

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: HOMO SAPIENS

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

GTCCATGTCC TTCCTGAAGG CGTAGAG 27

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: HOMO SAPIENS

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

TATGAATTCC TCTGCCTGCC CTGCCTCAAG AG 32

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: HOMO SAPIENS

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

CTAACACAGT CTCTCAAAGT 20

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: HOMO SAPIENS

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

ACTTTGAGAG ACTGTGTTAG 20

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: HOMO SAPIENS

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

TGGAGTGGCA ACTTCCAGGG 20

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: HOMO SAPIENS

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

CTCAGGGTCC TGTGGACAGC TCACCTAGCT GCA 33

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: HOMO SAPIENS

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

TGGACAGCTC ACCTAGCTGC A 21

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: HOMO SAPIENS

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

GGATTTCTGT TGTGTTTCCT 20

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: HOMO SAPIENS

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

TTGACACCTA CCAGGAGTTT 20

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: HOMO SAPIENS

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

CTAGAAGCCA CAGCTGCCCT 20

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: HOMO SAPIENS

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

GCGGATCCTT CCCAACCATT CCCTTATC 28

(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

TAAGAATTCG AGGGGTCACA GGGATGCCAC CCC 33

(2) INFORMATION FOR SEQ ID NO: 18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 191 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

```
Phe Pro Thr Ile Pro Leu Ser Arg Pro Phe Asp Asn Ala Met Leu Arg
1               5                   10                  15

Ala His Arg Leu His Gln Leu Ala Phe Asp Thr Tyr Gln Glu Phe Glu
            20                  25                  30

Glu Ala Tyr Ile Pro Lys Glu Gln Lys Tyr Ser Phe Leu Gln Asn Pro
        35                  40                  45

Gln Thr Ser Leu Cys Phe Ser Glu Ser Ile Pro Thr Pro Ser Asn Arg
    50                  55                  60

Glu Glu Thr Gln Gln Lys Ser Asn Leu Glu Leu Leu Cys Ile Ser Leu
65                  70                  75                  80

Leu Leu Ile Gln Ser Trp Leu Glu Pro Val Gln Phe Leu Arg Ser Val
                85                  90                  95

Phe Ala Asn Ser Leu Val Tyr Gly Ala Ser Asp Ser Asn Val Tyr Asp
            100                 105                 110

Leu Leu Lys Asp Leu Glu Glu Gly Ile Gln Thr Leu Met Gly Arg Leu
        115                 120                 125

Glu Asp Gly Ser Pro Arg Thr Gly Gln Ile Phe Lys Gln Thr Tyr Ser
    130                 135                 140

Lys Phe Asp Thr Asn Ser His Asn Asp Asp Ala Leu Leu Lys Asn Tyr
145                 150                 155                 160

Gly Leu Leu Tyr Ala Phe Arg Lys Asp Met Asp Lys Val Glu Thr Phe
                165                 170                 175

Leu Arg Ile Val Gln Cys Arg Ser Val Glu Gly Ser Cys Gly Phe
            180                 185                 190
```

(2) INFORMATION FOR SEQ ID NO: 19:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 191 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: HOMO SAPIENS

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

```
Phe Pro Thr Ile Pro Leu Ser Arg Pro Phe Asp Asn Ala Met Leu Arg
1               5                   10                  15

Ala His Arg Leu His Gln Leu Ala Phe Asp Thr Tyr Gln Glu Phe Glu
            20                  25                  30

Glu Ala Tyr Ile Pro Lys Glu Gln Lys Tyr Ser Phe Leu Gln Asn Pro
        35                  40                  45

Gln Thr Ser Leu Ala Phe Ser Glu Ser Ile Pro Thr Pro Ser Asn Arg
    50                  55                  60

Glu Glu Thr Gln Gln Lys Ser Asn Leu Glu Leu Leu Cys Ile Ser Leu
65                  70                  75                  80

Leu Leu Ile Gln Ser Trp Leu Glu Pro Val Gln Phe Leu Arg Ser Val
                85                  90                  95

Phe Ala Asn Ser Leu Val Tyr Gly Ala Ser Asp Ser Asn Val Tyr Asp
            100                 105                 110

Leu Leu Lys Asp Leu Glu Glu Gly Ile Gln Thr Leu Met Gly Arg Leu
        115                 120                 125

Glu Asp Gly Ser Pro Arg Thr Gly Gln Ile Phe Lys Gln Thr Tyr Ser
    130                 135                 140

Lys Phe Asp Thr Asn Ser His Asn Asp Asp Ala Leu Leu Lys Asn Tyr
145                 150                 155                 160

Gly Leu Leu Tyr Cys Phe Arg Lys Asp Met Asp Lys Val Glu Thr Phe
                165                 170                 175

Leu Arg Ile Val Gln Cys Arg Ser Val Glu Gly Ser Cys Gly Phe
            180                 185                 190
```

(2) INFORMATION FOR SEQ ID NO: 20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 191 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: HOMO SAPIENS

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

```
Phe Pro Thr Ile Pro Leu Ser Arg Pro Phe Asp Asn Ala Met Leu Arg
1               5                   10                  15

Ala His Arg Leu His Gln Leu Ala Phe Asp Thr Tyr Gln Glu Phe Glu
            20                  25                  30

Glu Ala Tyr Ile Pro Lys Glu Gln Lys Tyr Ser Phe Leu Gln Asn Pro
        35                  40                  45

Gln Thr Ser Leu Cys Phe Ser Glu Ser Ile Pro Thr Pro Ser Asn Arg
    50                  55                  60

Glu Glu Thr Gln Gln Lys Ser Asn Leu Glu Leu Leu Cys Ile Ser Leu
65                  70                  75                  80

Leu Leu Ile Gln Ser Trp Leu Glu Pro Val Gln Phe Leu Arg Ser Val
                85                  90                  95

Phe Ala Asn Ser Leu Val Tyr Gly Ala Ser Asp Ser Asn Val Tyr Asp
            100                 105                 110

Leu Leu Lys Asp Leu Glu Glu Gly Ile Gln Thr Leu Met Gly Arg Leu
        115                 120                 125

Glu Asp Gly Ser Pro Arg Thr Gly Gln Ile Phe Lys Gln Thr Tyr Ser
    130                 135                 140

Lys Phe Asp Thr Asn Ser His Asn Asp Asp Ala Leu Leu Lys Asn Tyr
145                 150                 155                 160

Gly Leu Leu Tyr Ala Phe Arg Lys Asp Met Asp Lys Val Glu Thr Phe
                165                 170                 175

Leu Arg Ile Val Gln Cys Arg Ser Val Glu Gly Ser Cys Gly Phe
            180                 185                 190
```

Claims

1. A mutant human growth hormone protein having an amino acid sequence as given in Figure 1 or subject to partial > replacement, insertion or deletion to such an extent as does not cause loss of enhanced affinity for the growth hormone receptor and decreased growth hormone activity.

2. A protein as claimed in Claim 1, having an amino acid sequence as given in Figure 2.

3. A protein as claimed in Claim 1, having an amino acid sequence as given in Figure 3.

4. A peptide constituting a portion of a protein as claimed in any one of Claims 1 to 3, which peptide constitutes a binding site for the receptor protein of said protein.

5. A deoxyribonucleotide having a base sequence as given in Figure 1, which encodes an amino acid sequence as claimed in Claim 1 or having a modified said base sequence encoding said amino acid sequence of Figure 1 subject to partial replacement, insertion or deletion to such an extent as does not cause loss of enhanced affinity

for the growth hormone receptor and decreased growth hormone activity.

6. A deoxyribonucleotide as claimed in Claim 5, having a base sequence which encodes the amino acid sequence of a protein as claimed in Claim 2 or Claim 3.

7. A deoxyribonucleotide having a base sequence which encodes a peptide as claimed in Claim 4.

8. An expression plasmid having a deoxyribonucleotide as claimed in any one of Claims 5 to 7 disposed downstream of a promoter.

9. A peptide or protein produced by expression by organism cells transformed with a plasmid as claimed in Claim 8.

10. An antibody immunoreactive with a mutant human growth hormone protein or peptide as claimed in any one of Claims 1 to 4.

11. An antibody as claimed in Claim 10, which is a monoclonal antibody.

12. A medicament for the treatment of gigantism or acromegaly which contains as an active ingredient a protein or peptide as claimed in any one of Claims 1 to 4.

13. A medicament for gene therapy which makes use of a deoxyribonucleotide as claimed in any one of Claims 5 to 7.

Fig. 1.

```
                9           18          27          36          45          54
5'TTC CCA ACC ATT CCC TTA TCC AGG CCT TTT GAC AAC GCT ATG CTC CGC GCC CAT
  -----------------------------------------------------------------------
   F   P   T   I   P   L   S   R   P   F   D   N   A   M   L   R   A   H

            63          72          81          90          99         108
  CGT CTG CAC CAG CTG GCC TTT GAC ACC TAC CAG GAG TTT GAA GAA GCC TAT ATC
  -----------------------------------------------------------------------
   R   L   H   Q   L   A   F   D   T   Y   Q   E   F   E   E   A   Y   I

           117         126         135         144         153         162
  CCA AAG GAA CAG AAG TAT TCA TTC CTG CAG AAC CCC CAG ACC TCC CTC TGT TTC
  -----------------------------------------------------------------------
   P   K   E   Q   K   Y   S   F   L   Q   N   P   Q   T   S   L   C   F

           171         180         189         198         207         216
  TCA GAG TCT ATT CCG ACA CCC TCC AAC AGG GAG GAA ACA CAA CAG AAA TCC AAC
  -----------------------------------------------------------------------
   S   E   S   I   P   T   P   S   N   R   E   E   T   Q   Q   K   S   N

           225         234         243         252         261         270
  CTA GAG CTG CTC TGC ATC TCC CTG CTG CTC ATC CAG TCG TGG CTG GAG CCC GTG
  -----------------------------------------------------------------------
   L   E   L   L   C   I   S   L   L   L   I   Q   S   W   L   E   P   V

           279         288         297         306         315         324
  CAG TTC CTC AGG AGT GTC TTC GCC AAC AGC CTG GTG TAC GGC GCC TCT GAC AGC
  -----------------------------------------------------------------------
   Q   F   L   R   S   V   F   A   N   S   L   V   Y   G   A   S   D   S

           333         342         351         360         369         378
  AAC GTC TAT GAC CTC CTA AAG GAC CTA GAG GAA GGC ATC CAA ACG CTG ATG GGG
  -----------------------------------------------------------------------
   N   V   Y   D   L   L   K   D   L   E   E   G   I   Q   T   L   M   G

           387         396         405         414         423         432
  AGG CTG GAA GAT GGC AGC CCC CGG ACT GGG CAG ATC TTC AAG CAG ACC TAC AGC
  -----------------------------------------------------------------------
   R   L   E   D   G   S   P   R   T   G   Q   I   F   K   Q   T   Y   S

           441         450         459         468         477         486
  AAG TTC GAC ACA AAC TCA CAC AAC GAT GAC GCA CTA CTC AAG AAC TAC GGG CTG
  -----------------------------------------------------------------------
   K   F   D   T   N   S   H   N   D   D   A   L   L   K   N   Y   G   L

           495         504         513         522         531         540
  CTC TAC TGC TTC AGG AAG GAC ATG GAC AAG GTC GAG ACA TTC CTG CGC ATC GTG
  -----------------------------------------------------------------------
   L   Y   C   F   R   K   D   M   D   K   V   E   T   F   L   R   I   V

           549         558         567
  CAG TGC CGC TCT GTG GAG GGC AGC TGT GGC TTC 3'
  -------------------------------------------
   Q   C   R   S   V   E   G   S   C   G   F
```

Fig. 2.

```
            9           18          27          36          45          54
5 'TTC CCA ACC ATT CCC TTA TCC AGG CCT TTT GAC AAC GCT ATG CTC CGC GCC CAT
   ------------------------------------------------------------------------
    F   P   T   I   P   L   S   R   P   F   D   N   A   M   L   R   A   H

           63          72          81          90          99         108
   CGT CTG CAC CAG CTG GCC TTT GAC ACC TAC CAG GAG TTT GAA GAA GCC TAT ATC
   ------------------------------------------------------------------------
    R   L   H   Q   L   A   F   D   T   Y   Q   E   F   E   E   A   Y   I

          117         126         135         144         153         162
   CCA AAG GAA CAG AAG TAT TCA TTC CTG CAG AAC CCC CAG ACC TCC CTC GCT TTC
   ------------------------------------------------------------------------
    P   K   E   Q   K   Y   S   F   L   Q   N   P   Q   T   S   L   A   F

          171         180         189         198         207         216
   TCA GAG TCT ATT CCG ACA CCC TCC AAC AGG GAG GAA ACA CAA CAG AAA TCC AAC
   ------------------------------------------------------------------------
    S   E   S   I   P   T   P   S   N   R   E   E   T   Q   Q   K   S   N

          225         234         243         252         261         270
   CTA GAG CTG CTC TGC ATC TCC CTG CTG CTC ATC CAG TCG TGG CTG GAG CCC GTG
   ------------------------------------------------------------------------
    L   E   L   L   C   I   S   L   L   L   I   Q   S   W   L   E   P   V

          279         288         297         306         315         324
   CAG TTC CTC AGG AGT GTC TTC GCC AAC AGC CTG GTG TAC GGC GCC TCT GAC AGC
   ------------------------------------------------------------------------
    Q   F   L   R   S   V   F   A   N   S   L   V   Y   G   A   S   D   S

          333         342         351         360         369         378
   AAC GTC TAT GAC CTC CTA AAG GAC CTA GAG GAA GGC ATC CAA ACG CTG ATG GGG
   ------------------------------------------------------------------------
    N   V   Y   D   L   L   K   D   L   E   E   G   I   Q   T   L   M   G

          387         396         405         414         423         432
   AGG CTG GAA GAT GGC AGC CCC CGG ACT GGG CAG ATC TTC AAG CAG ACC TAC AGC
   ------------------------------------------------------------------------
    R   L   E   D   G   S   P   R   T   G   Q   I   F   K   Q   T   Y   S

          441         450         459         468         477         486
   AAG TTC GAC ACA AAC TCA CAC AAC GAT GAC GCA CTA CTC AAG AAC TAC GGG CTG
   ------------------------------------------------------------------------
    K   F   D   T   N   S   H   N   D   D   A   L   L   K   N   Y   G   L

          495         504         513         522         531         540
   CTC TAC TGC TTC AGG AAG GAC ATG GAC AAG GTC GAG ACA TTC CTG CGC ATC GTG
   ------------------------------------------------------------------------
    L   Y   C   F   R   K   D   M   D   K   V   E   T   F   L   R   I   V

          549         558         567
   CAG TGC CGC TCT GTG GAG GGC AGC TGT GGC TTC  3 '
   --------------------------------------------
    Q   C   R   S   V   E   G   S   C   G   F
```

Fig. 3.

```
           9           18          27          36          45          54
5 'TTC CCA ACC ATT CCC TTA TCC AGG CCT TTT GAC AAC GCT ATG CTC CGC GCC CAT
   ------------------------------------------------------------------------
    F   P   T   I   P   L   S   R   P   F   D   N   A   M   L   R   A   H

           63          72          81          90          99         108
   CGT CTG CAC CAG CTG GCC TTT GAC ACC TAC CAG GAG TTT GAA GAA GCC TAT ATC
   ------------------------------------------------------------------------
    R   L   H   Q   L   A   F   D   T   Y   Q   E   F   E   E   A   Y   I

          117         126         135         144         153         162
   CCA AAG GAA CAG AAG TAT TCA TTC CTG CAG AAC CCC CAG ACC TCC CTC TGT TTC
   ------------------------------------------------------------------------
    P   K   E   Q   K   Y   S   F   L   Q   N   P   Q   T   S   L   C   F

          171         180         189         198         207         216
   TCA GAG TCT ATT CCG ACA CCC TCC AAC AGG GAG GAA ACA CAA CAG AAA TCC AAC
   ------------------------------------------------------------------------
    S   E   S   I   P   T   P   S   N   R   E   E   T   Q   Q   K   S   N

          225         234         243         252         261         270
   CTA GAG CTG CTC TGC ATC TCC CTG CTG CTC ATC CAG TCG TGG CTG GAG CCC GTG
   ------------------------------------------------------------------------
    L   E   L   L   C   I   S   L   L   L   I   Q   S   W   L   E   P   V

          279         288         297         306         315         324
   CAG TTC CTC AGG AGT GTC TTC GCC AAC AGC CTG GTG TAC GGC GCC TCT GAC AGC
   ------------------------------------------------------------------------
    Q   F   L   R   S   V   F   A   N   S   L   V   Y   G   A   S   D   S

          333         342         351         360         369         378
   AAC GTC TAT GAC CTC CTA AAG GAC CTA GAG GAA GGC ATC CAA ACG CTG ATG GGG
   ------------------------------------------------------------------------
    N   V   Y   D   L   L   K   D   L   E   E   G   I   Q   T   L   M   G

          387         396         405         414         423         432
   AGG CTG GAA GAT GGC AGC CCC CGG ACT GGG CAG ATC TTC AAG CAG ACC TAC AGC
   ------------------------------------------------------------------------
    R   L   E   D   G   S   P   R   T   G   Q   I   F   K   Q   T   Y   S

          441         450         459         468         477         486
   AAG TTC GAC ACA AAC TCA CAC AAC GAT GAC GCA CTA CTC AAG AAC TAC GGG CTG
   ------------------------------------------------------------------------
    K   F   D   T   N   S   H   N   D   D   A   L   L   K   N   Y   G   L

          495         504         513         522         531         540
   CTC TAC GCC TTC AGG AAG GAC ATG GAC AAG GTC GAG ACA TTC CTG CGC ATC GTG
   ------------------------------------------------------------------------
    L   Y   A   F   R   K   D   M   D   K   V   E   T   F   L   R   I   V

          549         558         567
   CAG TGC CGC TCT GTG GAG GGC AGC TGT GGC TTC 3 '
   -------------------------------------------
    Q   C   R   S   V   E   G   S   C   G   F
```

Fig. 4a

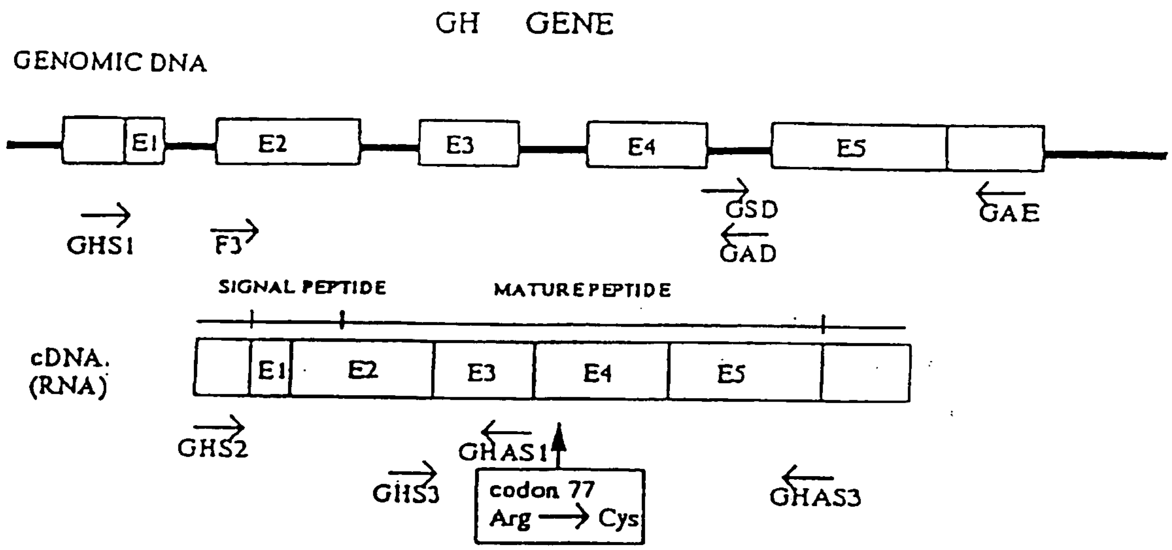

GH GENE
GENOMIC DNA
E1
E2
E3
E4
E5
GHS1
F3
GSD
GAD
GAE
SIGNAL PEPTIDE
MATURE PEPTIDE
cDNA.
(RNA)
GHS2
GHAS1
GHS3
codon 77
Arg ⟶ Cys
GHAS3

Fig. 4b

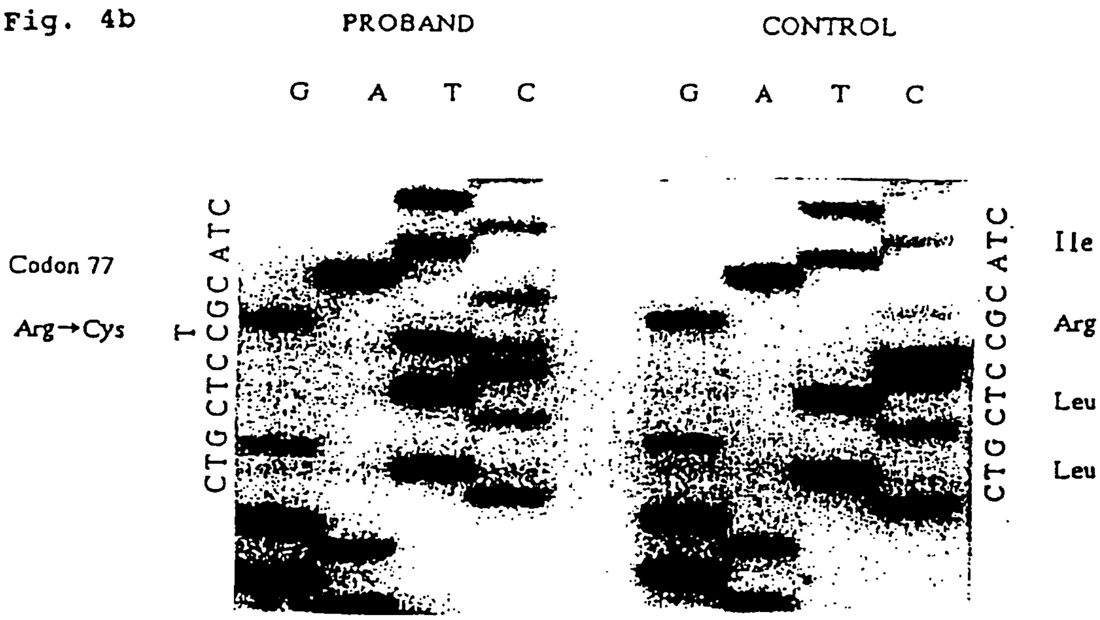
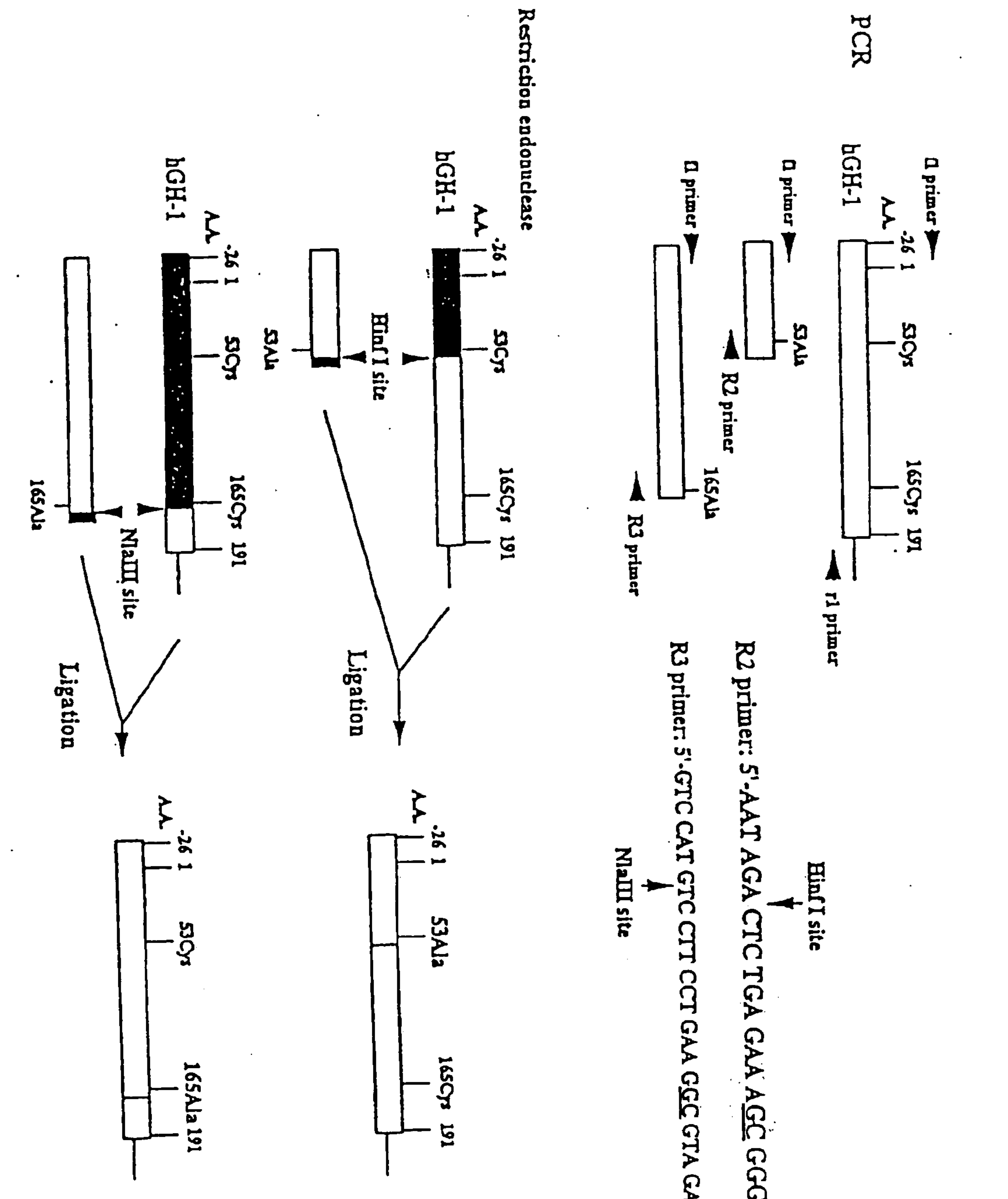

PROBAND
CONTROL
G A T C
G A T C
Codon 77
Arg→Cys
T
CTG CTC CGC ATC
CTG CTC CGC ATC
Ile
Arg
Leu
Leu

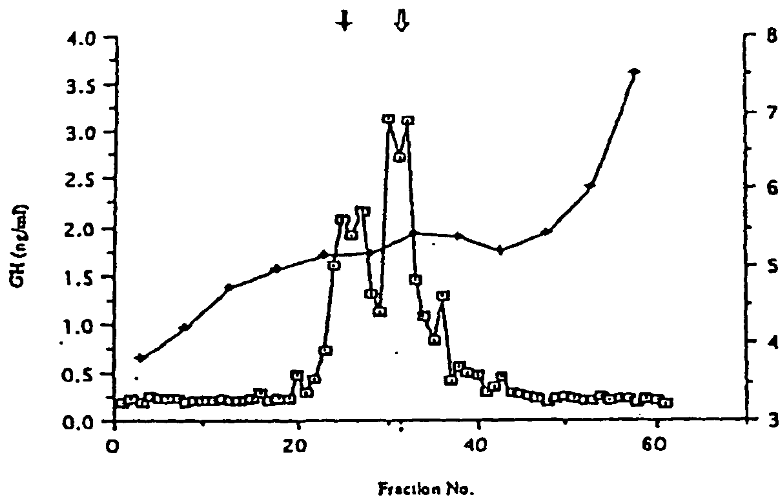
Fig. 5



PCR
r1 primer
bGH-1
-26
1
53Cys
165Cys
191
r1 primer
53Ala
R2 primer
165Ala
R3 primer
R2 primer: 5'-AAT AGA CTC TGA GAA AGC GGG-3'
HinfI site
R3 primer: 5'-GTC CAT GTC CTT CCT GAA GGC GTA GAG -3'
NlaIII site
Restriction endonuclease
HinfI site
Ligation
NlaIII site
53Ala
165Ala

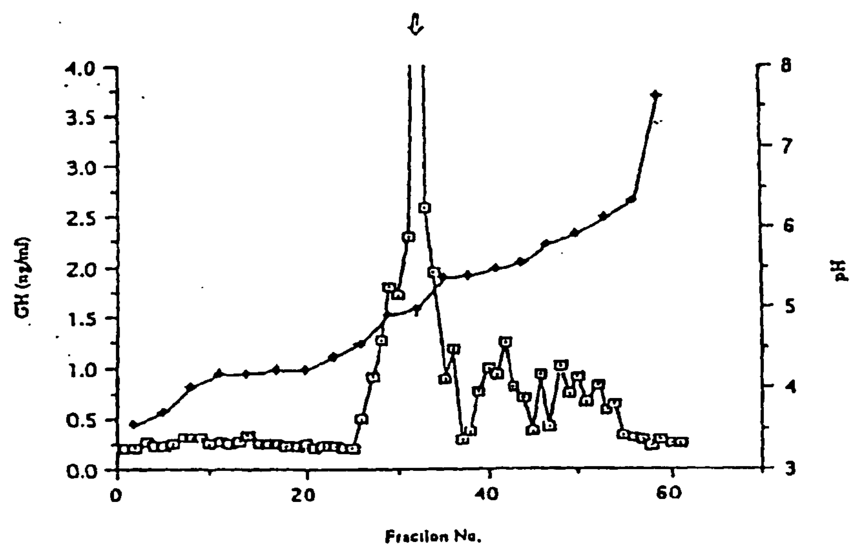
Fig. 6

a.



GH (ng/ml)
pH
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
8
7
6
5
4
3
0
20
40
60
Fraction No.

b.



GH (ng/ml)
pH
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
8
7
6
5
4
3
0
20
40
60
Fraction No.

Fig. 7a

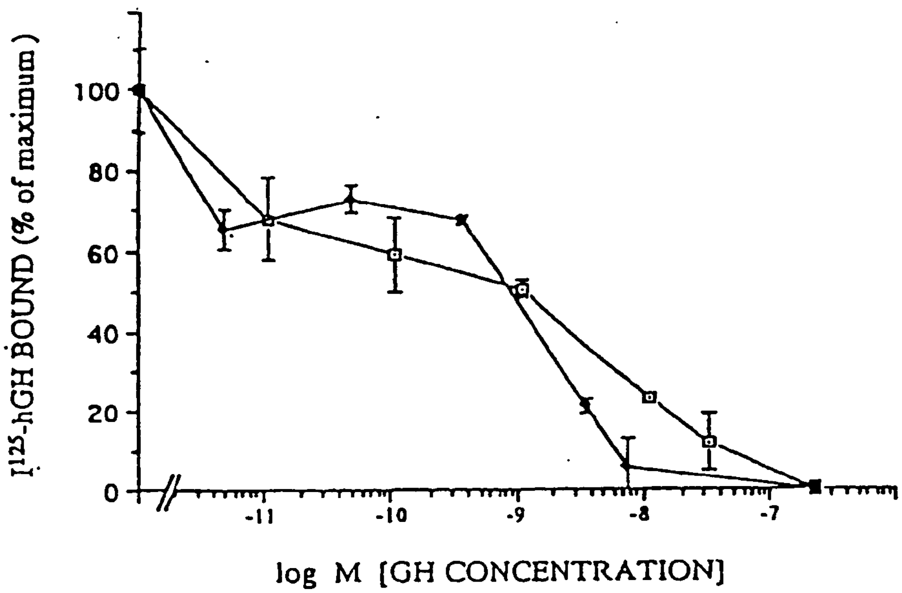

I125-hGH BOUND (% of maximum)
100
80
60
40
20
0
-11
-10
-9
-8
-7
log M [GH CONCENTRATION]

Fig. 7b

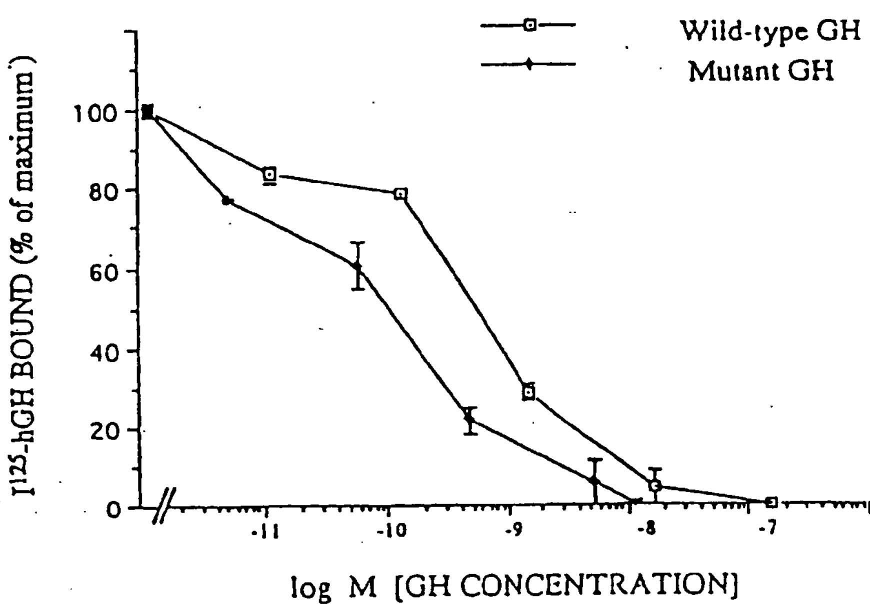

Wild-type GH
Mutant GH
I125-hGH BOUND (% of maximum)
100
80
60
40
20
0
-11
-10
-9
-8
-7
log M [GH CONCENTRATION]

Fig. 8a

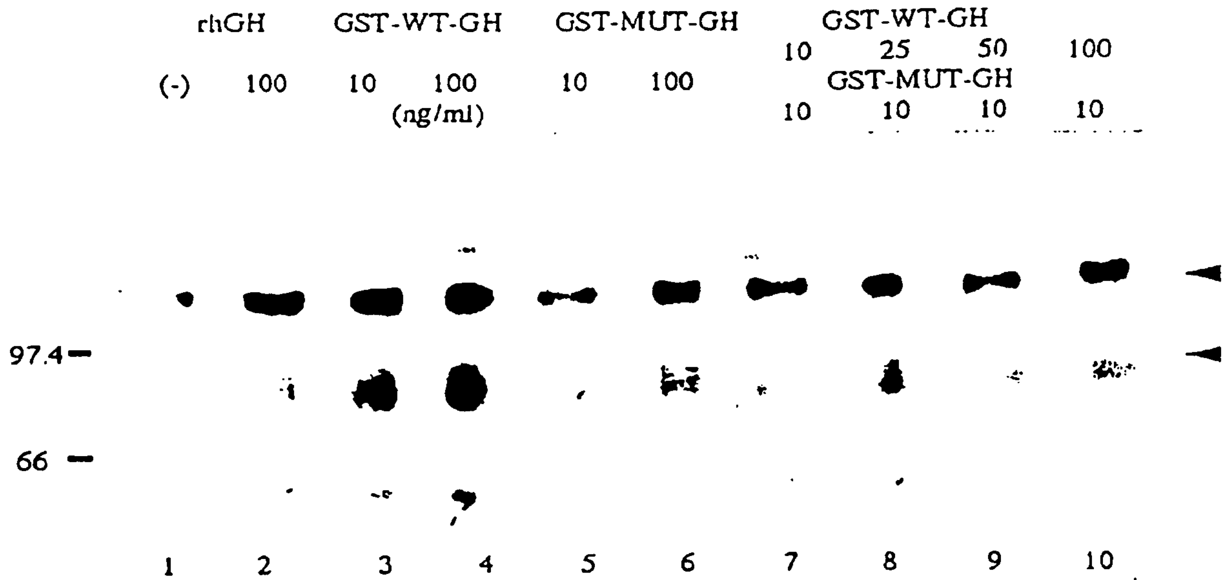
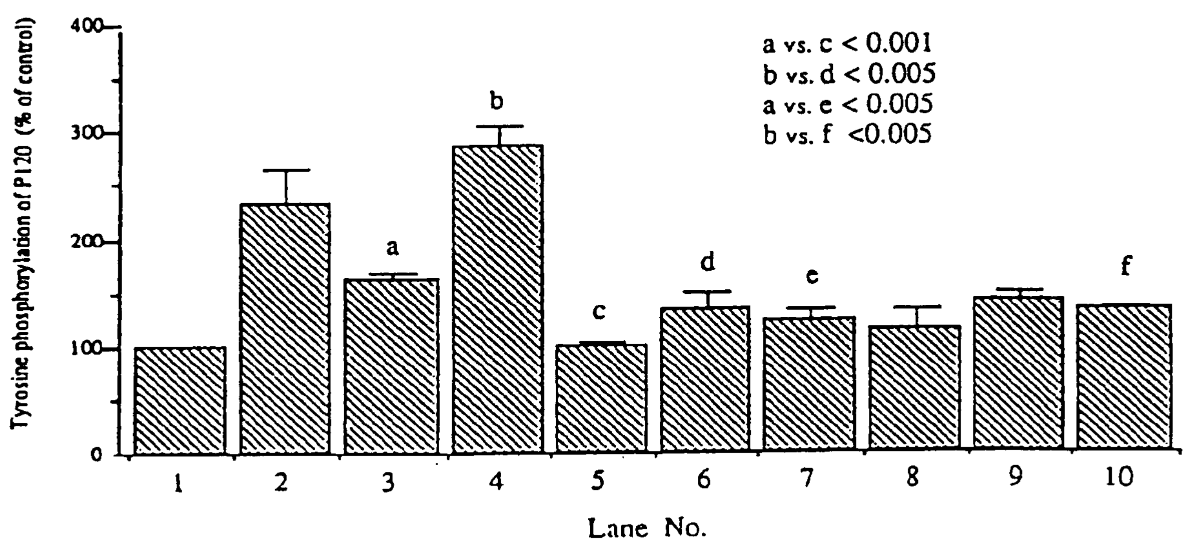

rhGH
GST-WT-GH
GST-MUT-GH
GST-WT-GH
10 25 50 100
GST-MUT-GH
10 10 10 10
(-) 100 10 100 10 100
(ng/ml)
97.4
66
1 2 3 4 5 6 7 8 9 10

Fig. 8b


Tyrosine phosphorylation of P120 (% of control)
400
300
200
100
0
a vs. c < 0.001
b vs. d < 0.005
a vs. e < 0.005
b vs. f <0.005
a
b
c
d
e
f
1 2 3 4 5 6 7 8 9 10
Lane No.

European Patent Office

EUROPEAN SEARCH REPORT

Application Number
EP 97 30 0902

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
X	WO 92 19736 A (OHIO UNIVERSITY) 12 November 1992 * the whole document * ---	4,7-13	C12N15/11 C07K14/61 A61K38/27 A61K48/00 C07K16/26
X	EP 0 319 049 A (BIOTECHNOLOGY GENERAL CORPORATION) 7 June 1989 * the whole document * ---	4,7-13	
X,D	BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, vol. 172, no. 1, 15 October 1990, ORLANDO, FL US, pages 357-363, XP002032925 E UCHIDA ET AL.: "An active site of growth hormone for eliciting the differentiation of preadipose 3T3-F442A cells to adipose cells" * the whole document * -----	4	

TECHNICAL FIELDS SEARCHED (Int.Cl.6)

C12N
C07K
A61K

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	12 June 1997	Masturzo, P

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
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